EPA/OPP MICROBIOLOGY LABORATORY
ESC, Ft. Meade, MD

Standard Operating Procedure
for
Neutralization Confirmation Procedure for Products Evaluated with the AOAC Use Dilution Test and the AOAC Germicidal Spray Product Test (*Staphylococcus aureus* and *Pseudomonas aeruginosa*)

SOP Number: MB-17-00

Date Revised: 1-28-06

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1.0 SCOPE AND APPLICATION:

1.1 The neutralization of the active ingredients found in antimicrobial products is one of the most important steps in efficacy testing. A neutralizing agent is used to inactivate the product's active ingredients, a process essential to achieving the desired contact time. In addition, the neutralizer itself or in combination with the recovery medium must not exhibit bacteriostatic activity against the test microbes. Bacteriostatic activity may bias the outcome of an efficacy evaluation.

1.2 This SOP describes methodology which will be used to determine the effectiveness of neutralizers specified for hospital disinfectant efficacy testing. The Neutralization Confirmation Procedure is a carrier-based method (sterile carriers) which simulates the test conditions, but is designed to quantitatively assess the effectiveness of neutralizers across a broad range of microbe concentrations.

1.3 *Staphylococcus aureus* (ATCC #6538) and *Pseudomonas aeruginosa* (ATCC #15442) will be the test microbes in hospital disinfectant efficacy evaluation, and will be used in the neutralization testing.

1.4 General Description of the Assay.

1.4.1 The test conditions specified for product testing (e.g., H_2O hardness, Use Dilution, pH, Organic Soil, Neutralizer, Contact Time, Temperature) must also be followed for the neutralization confirmation assay. Record the test information on the appropriate Information Sheet for the Neutralization Confirmation Assay (see 16.0).

1.4.2 This assay is designed to simulate the conditions of the AOAC UDT and AOAC GSPT; however, sterile carriers are used instead of inoculated carriers. Diluted *S. aureus* or *P. aeruginosa* inoculum is added directly to the various sets of subculture media tubes (see Table 1). The inoculum is quantified by plating on a suitable agar such as TSA. This provides for a quantitative approach to assessing the effectiveness of the neutralizer and any bacteriostatic action resulting from the neutralizer itself or neutralizer x disinfectant interactions.

1.5 This method can also be used to determine the effectiveness of an alternative neutralizer, one not specified in the test parameters.

1.6 It is preferable to perform the neutralization assay concurrently with product testing; however, an independent, stand-alone assay may also be performed in the event that the potential success of the neutralizer is questionable.

2.0 DEFINITIONS:

2.1 AOAC = AOAC INTERNATIONAL

2.2 AOAC UDT = AOAC Use Dilution Test

2.3 AOAC GSPT = AOAC Germicidal Spray Products Test

2.3 CFU = Colony Forming Unit

2.4 PBDW = Phosphate Buffered Dilution Water

2.5 TSA = Tryptic Soy Agar

2.6 DI = Deionized Water

3.0 HEALTH AND SAFETY:

3.1 All manipulations of the test organisms (*S. aureus* and *P. aeruginosa*) are required to be performed in accordance with biosafety practices stipulated in the SOP MB-01, Biosafety in the Laboratory. Biosafety level 2 practices will be followed for tests involving *S. aureus* and *P. aeruginosa*.

3.2 Disinfectants may contain a number of different active ingredients, such as heavy metals, aldehydes, peroxides, and phenol. Latex gloves and other personal protective clothing or devices must be worn during the handling of these items for purposes of activation or dilution, or efficacy testing. A chemical fume hood or other containment equipment is employed when performing tasks with concentrated products.

4.0 CAUTIONS:

4.1 To ensure the stability of the test disinfectant, prepare the disinfectant dilutions within three hours of the disinfectant treatment step unless test parameters specify otherwise.

4.2 Strict adherence to the protocol is necessary for validity of test results.

4.3 Use aseptic procedures for all test procedures involving manipulations of the test organisms and associated test components.

5.0 INTERFERENCES:

5.1 For each neutralizer and medium tested per study, one batch (preparation) should be used for all treatment and control groups. Differences in performance (quality)

between batches of media may lead to misleading neutralization results.

6.0 PERSONNEL QUALIFICATIONS:

6.1 Personnel are required to be knowledgeable of the procedures in this SOP. Documentation of training and familiarization with this SOP can be found in the training file for each employee.

7.0 SPECIAL APPARATUS AND MATERIALS: None

8.0 INSTRUMENT OR METHOD CALIBRATION:

8.1 Refer to the laboratory equipment calibration and maintenance SOPs (SOP EQ series) for details on method and frequency of calibration.

9.0 SAMPLE HANDLING AND STORAGE:

9.1 Disinfectants are stored according to manufacturers' recommendations or at room temperature if the product label or testing parameters do not identify a storage temperature. Those disinfectants requiring activation or dilution prior to use will only be activated or diluted within three hours of testing unless test parameters specify otherwise.

10.0 PROCEDURE AND ANALYSIS:

10.1 Preparation, Dilution, and Enumeration of Inoculum.

10.1.1 For Assay Simulating the AOAC Use Dilution Test. The inoculum is prepared according to SOP MB-05, AOAC Use Dilution Test for Testing Disinfectants, sections 10.2 and 10.3. In section 10.3, do not proceed past the point where four tubes of inoculum are prepared (i.e., do not inoculate carriers).

10.1.2 For Assay Simulating the AOAC Germicidal Spray Product Test. The inoculum is prepared according to SOP MB-06, AOAC Germicidal Spray Product Test for Testing Disinfectants, section 10.3. In section 10.3, do not proceed past the point where four tubes of inoculum are prepared (i.e., do not inoculate carriers).

10.1.3 If the product test conditions include the addition of an organic soil load to the inoculum, then the neutralization assay will be performed with the organic soil load added to the inoculum as per SOP MB-05, section 10.3.2 or SOP MB-06, section 10.3.11. Otherwise, the inoculum should be prepared without the addition of an organic soil load.

10.1.4 Initiate serial ten-fold dilutions of the inoculum by pipetting 1 mL of the inoculum into 9 mL of PBDW or sterile deionized water. Four dilutions, (1.0×10^{-5}, 1.0×10^{-6}, 1.0×10^{-7}, and 1.0×10^{-8}) will be used to inoculate the neutralizer and subculture media tubes described below. The target number of cells to be delivered (section 10.3.9) is 5-100 CFU/mL; this level should be seen in one of the two highest dilutions.

10.1.5 To estimate CFU/mL, plate (pour plate or spread plate method) each of the four dilutions in duplicate on TSA agar. Briefly vortex each dilution tube prior to plating. See 10.1.7 for pour plate method and 10.1.8 for spread plate method.

10.1.6 Record the dilution and plating information on the appropriate Neutralization Confirmation Assay Serial Dilution/Plating Tracking Form and the Neutralization Confirmation Assay Inoculum Enumeration Form (see 16.0).

10.1.7 Pour plate method: the TSA agar is prepared and tempered (approx. 1 hr) to 45-50ºC in a waterbath prior to use. Tempered TSA agar is added to the plate after the addition of the appropriate dilution, and swirled to spread the inoculum.

10.1.8 Spread plate method: allow refrigerated plates to come to room temperature prior to use. To spread dilutions evenly over the surface of the agar, tilt the plate back and forth or, if necessary, use a glass spreading rod and plate spinner.

10.1.9 Incubate plates at 37±1ºC for 24-48 hours. Count colonies with aid of a plate counter. Plates that have colony counts over 300 can be estimated or labeled TNTC. Record the counts on the Neutralization Confirmation Assay Inoculum Enumeration Form (see 16.0).

10.2 Product Sample Preparation.

10.2.1 Follow guidelines for disinfectant sample preparation provided in the appropriate test method SOP.

10.3 Performing the Assay. The following instructions apply to the analysis of one neutralizer with one carrier type, and one test organism.

10.3.1 Each assay will require four sterile carriers per organism. Use the carrier type required for the specific test. Carriers are prepared according to SOP MB-03, Screening Carriers Used in Disinfectant Efficacy Testing.

10.3.1.1 For liquid products: Sterile stainless steel carriers will be removed from the asparagine solution, placed onto filter paper in sterile glass Petri dishes, and dried for 40 minutes per SOP MB-05.

10.3.1.2 For spray products: Before conducting the neutralization assay, the analyst must practice applying the spray product to sterile glass slides to determine the product's level of dispersion. If the product beads up and rolls off of the slide rather than completely covering the glass slide as it would in a typical efficacy evaluation (with inoculated slides), the analyst should apply 10 ul of an organic material (e.g., broth used for culturing test organism, 5% horse serum in sterile deionized water) onto the surface of each sterile glass slide and spread it with a sterile loop. Dry the slides for 40 minutes per SOP MB-06. Once drying is complete, apply the spray product again to determine if product dispersion is improved. Based on previous observations in our laboratory, the addition of an organic substance to the surface of the slide increases product dispersion on glass slides. If the application of an organic substance is deemed necessary to perform the test, apply the substance, dry the slides for 40 minutes as per SOP MB-06, and conduct the neutralization assay. If no organic substance is deemed necessary, no drying of the sterile glass slides is necessary prior to conducting the assay.

10.3.2 The product must be applied to the sterile carriers according to specific instruction provided in the test parameters (e.g., use dilution, spray distance, spray period, contact time).

10.3.3 Per test, one test per organism, four of the carriers are exposed to the disinfectant for the specified contact time in the same manner as product efficacy testing. Record the carrier transfer information on the Neutralization Confirmation Assay: Time Recording Sheet for Carrier Transfers

10.3.4 After the last carrier of a set (4 total carriers) has been treated with the disinfectant, and the contact time is complete, aseptically transfer carriers in order in a timed fashion into tubes containing the specified neutralizer, in the same manner as product efficacy testing. Drain excess liquid from the carrier prior to the transfer. This set of neutralizer tubes (4 total tubes) will represent the **Neutralizer-Primary Subculture** Treatment. Each tube will be inoculated with one mL of each of the four inoculum dilutions as indicated in Table 1 and section 10.3.9.

Note: For spray products, the amount of neutralizer is 20 mL per tube (38 x 100 mm Bellco tubes) compared to 10 mL (20 x 150 mm tubes) used in the test method for liquid products.

10.3.5 Following the last carrier transfer into the neutralizer tube, incubate the neutralizer tubes at 37± 1ºC for a minimum of 30 minutes. Then transfer each carrier in order into a culture tube containing the secondary subculture medium. This portion of the assay is not timed, but the transfers should be made as soon as possible after the end of the 30 minute incubation. This set of tubes (4 total tubes) will represent the **Secondary Subculture** Treatment. Each tube will be inoculated with one mL of each of the four inoculum dilutions as indicated in Table 1 and section 10.3.9.

10.3.6 Repeat the assay for the second test organism, if required.

10.3.7 Inoculated Controls.

The **Neutralizer-Primary** Inoculated Control contains four tubes of fresh, unexposed (to disinfectant) neutralizer-primary media.

The **Secondary Subculture** Inoculated Control contains four tubes of secondary subculture media.

It is highly desirable that the preparation (media preparation number) of each medium be the same as used in the treatments. Each tube will be inoculated with one of four inoculum dilutions as indicated in Table 1 and section 10.3.9.

10.3.8 Uninoculated Controls.

Neutralizer-Primary and **Secondary Subculture** uninoculated controls. One tube each of uninoculated neutralizer and secondary subculture media will be included in the test and incubated with the other tubes. Sterility of carriers must be confirmed either in advance or concurrently with testing by adding the carrier to a tube of 10 mL fluid thioglycollate medium and incubating at 37±1ºC for 5-7 days.

10.3.9 Inoculating the Tubes. Inoculate each *inoculated* treatment and control tube with 1 mL of the diluted inoculum as indicated in Table 1. **Inoculate the media following the transfer of all carriers into the secondary subculture media.**

Table 1. Inoculation of Treatment and Control Groups with Dilutions of the Test Organism*

Neutralizer-Primary Subculture Treatment	Secondary Subculture Treatment (with Carrier)	Neutralizer-Primary Inoculated Control	Secondary Subculture Inoculated Control	Neutralizer-Primary & Secondary Subculture Uninoculated Controls
1 mL of 10^{-5} → Tube 1 1 mL of 10^{-6} → Tube 2 1 mL of 10^{-7} → Tube 3 1 mL of 10^{-8} → Tube 4	1 mL of 10^{-5} → Tube 1 1 mL of 10^{-6} → Tube 2 1 mL of 10^{-7} → Tube 3 1 mL of 10^{-8} → Tube 4	1 mL of 10^{-5} → Tube 1 1 mL of 10^{-6} → Tube 2 1 mL of 10^{-7} → Tube 3 1 mL of 10^{-8} → Tube 4	1 mL of 10^{-5} → Tube 1 1 mL of 10^{-6} → Tube 2 1 mL of 10^{-7} → Tube 3 1 mL of 10^{-8} → Tube 4	**Not inoculated** 1 Tube of Neutralizer 1 Tube of Secondary Subculture Media

*1 x 10^{-5} through 1 x 10^{-8}; based on an approx. starting suspension of 10^7 to 10^8 CFU/mL

10.3.10 Incubate tubes for 48±2 hours at 37±1ºC.

10.4 Results are recorded as + (growth) or 0 (no growth). Record results on the appropriate Neutralization Confirmation Assay Results Form (see 16.0). Confirmation testing of the growth will be performed as listed in section 10.5.

10.5 Identification and Confirmation Testing.

10.5.1 A minimum of one positive tube per treatment and control, if available, should be confirmed using Gram staining. If further confirmation is deemed necessary, selective media and VITEK analysis may be used.

For each treatment and control group, select the tube with the highest dilution showing growth (inoculated with the dilution with fewest CFU/mL delivered) and conduct confirmation testing on a sample of the growth.

10.5.2 Record confirmation results on the Neutralization Confirmation Assay Microbe Confirmation Sheet (see 16.0).

10.6 Interpretation of Results.

10.6.1 Plate count data. One of the four dilutions plated should provide counts within the approximate target range, 5-100 CFU/mL.

10.6.1.1 Note: The lack of complete neutralization of the disinfectant or bacteriostatic activity of the neutralizer itself may be masked when a high level of inoculum is added to the subculture tubes.

10.6.2 Controls. Growth in the **Secondary Subculture** Inoculated Control verifies the presence of the test microbe, performance of the media, and provides a basis for comparison of growth in the neutralizer and subculture treatment tubes. *No growth or only growth in tubes which*

received high levels of inoculum (e.g., a dilution with plate counts which are too numerous to count) indicates poor media performance. Growth in the **Neutralizer-Primary** Inoculated Control should be comparable to the Secondary Subculture inoculated Control if the neutralizer is the same as the secondary subculture media.

There may be cases when the neutralizer is significantly different from the secondary subculture media. In these cases, growth may not be comparable to the Secondary Subculture inoculated Control.

The **Neutralizer-Primary** and **Secondary Subculture** uninoculated control tubes are used to determine sterility, and must show no growth for the test to be valid.

10.6.3 Treatments. The occurrence of growth in the **Neutralizer-Primary Subculture** and **Secondary Subculture** treatment tubes are used to assess the effectiveness of the neutralizer. The neutralizer itself or in combination with the recovery (subculture) medium may exhibit bacteriostatic activity against the test microbe. *No growth or growth only in tubes which received a high Level of inoculum (e.g., the dilution with plate counts which are too numerous to count) indicates poor neutralization and/or presence of bacteriostatic properties of the neutralizer or neutralizer-disinfectant interactions. For the neutralizer to be deemed effective, growth must occur in the **Secondary Subculture** treatment tubes which received lower levels of inoculum (e.g., 5-100 CFU/mL).*

11.0 DATA ANALYSIS/CALCULATIONS:

11.1 Plate counts are enumerated and CFU/mL is calculated based on the average of countable plates.

11.2 The evaluation of the data to determine whether a bacteriostatic effect is present is described in Section 10.6.

12.0 DATA MANAGEMENT/RECORDS MANAGEMENT:

12.1 Data will be recorded promptly, legibly, and in indelible ink on the forms indicated in section 16.0. Completed forms are archived in notebooks kept in secure file cabinets in D217. Only authorized personnel have access to the secure files. Archived data is subject to OPP's official retention schedule contained in SOP ADM-03, Records and Archives.

13.0 QUALITY CONTROL:

13.1 For quality control purposes, the required information is documented on the appropriate form(s) (see 16.0).

14.0 NONCONFORMANCE AND CORRECTIVE ACTION:

14.1 Strict adherence to the protocol is necessary for the validity of the test results. Any deviation from the standard protocol must be brought to the study director's attention and recorded in the raw data, and an explanation for the deviation given. The deviation and reason for it must be documented on the GLP Compliance form in the final report.

15.0 REFERENCES:

15.1 Official Methods of Analysis. 1990. 15th Ed., Association of Official Analytical Chemists, Arlington, VA (Methods 955.15, 964.02, and 961.02)

16.0 FORMS AND DATA SHEETS:

16.1 Neutralization Confirmation Assay: Time Recording Sheet for Carrier Inoculation Steps

16.2 Neutralization Confirmation Assay: Time Recording Sheet for Carrier Transfers

16.3 Information Sheet for the Neutralization Confirmation Assay

16.4 Neutralization Confirmation Assay: Results Form

16.5 Neutralization Confirmation Assay: Microbe Confirmation Sheet

16.6 Neutralization Confirmation Assay: Serial Dilution/Plating Tracking Form

16.7 Neutralization Confirmation Assay: Inoculum Enumeration Form

Neutralization Confirmation Assay: Time Recording Sheet for Carrier Inoculation Steps

OPP Microbiology Laboratory

TEST INFORMATION/Confirmed by:_____	
Test Date	
Type of Test	
Product Reg. No.	
Product Name	
Sample No(s).	
Organism	

Initials/Date	Test ID	Inoculum Settle Time*		Carrier Loading Time*+		Carrier Dry Time*	
		Start Time	End Time	Start Time	End Time	Start Time	End Time
		/	/	/	/	/	/
		/	/	/	/	/	/
		/	/	/	/	/	/
		/	/	/	/	/	/
		/	/	/	/	/	/
		/	/	/	/	/	/

* Recorded from laboratory clock/and timer.
+ Use to record time for application of organic material to sterile glass slides.

Neutralization Confirmation Assay: Time Recording Sheet for Carrier Transfers

OPP Microbiology Laboratory

TEST INFORMATION/Confirmed by:_____	
Test Date	
EPA Reg. No.	
Product Name	
Sample No(s).	
Organism(s)	
Neutralizer(s)	
Carrier Type	
Test Type	

Initials /date	Disinfectant Tube No.	Carrier No.	Carrier Drop Start Time for first carrier into the disinfectant		Carrier Drop End Time for last carrier into the neutralizer		First Carrier Transfer (into secondary media)
			Clock	Timer	Clock	Timer	Start Time[1]
	1-4	1-4					
Comments:							

1=Carrier transfer into secondary subculture (time elapsed after last carrier dropped in primary); taken from clock

Information Sheet for the Neutralization Confirmation Assay

OPP Microbiology Laboratory

TEST INFORMATION/Confirmed by:_____			
EPA Reg. No.		SOP	
Product Name		Test Date	
Sample No.		Neutralizer	
Lot No.		Comments:	
Expiration Date		Test Type: ___AOAC Use Dilution Test ___AOAC Germicidal Spray Products Test	

TEST PARAMETERS/Confirmed by:_____			
H_2O Hardness ($CaCO_3$) ppm	Specified	Titrated (Buret)/Date/Init	HACH/Date/Init
		/ /	/ /
Use Dilution	Specified	As Prepared/Date/Initials	
		/ /	
Organic Soil	Specified	As Prepared/Date/Init.	
Neutralizer	Specified		
Temperature (°C)	Specified	Chiller Unit Display	Test Tube Waterbath
		Before: After:	Before: After:
Contact Time	Specified	As Tested	
Other Parameters	Specified		
Carriers (not inoculated)		Control #	Preparation #
	Type:		

TEST MICROBE INFORMATION/Confirmed by:_____				
Test Microbe		48-54 Hour Culture		
Org. Control No.			Initiated	Harvested
Avg. CFU/Carrier	Not Applicable	Date/Time		

REAGENT/MEDIA INFORMATION/Confirmed by:_____			
Reagent/Media	Prep No.	Reagent/Media	Prep No.

Neutralization Confirmation Assay: Results Form
OPP Microbiology Laboratory

TEST INFORMATION/Confirmed by:_____			
EPA Reg. No.		Test Date	
Product Name		Neutralizer	
Sample No.		Comments:	
Test Type			

CARRIER INFORMATION/ Confirmed by: _____		
Carrier Spray Time Interval	Carrier Set	Analyst

TEST RESULTS*: Date Recorded/Initials:_____				
Treatments/Controls	Inoculum Dilutions			
	1.0×10^{-5}	1.0×10^{-6}	1.0×10^{-7}	1.0×10^{-8}
Neutralizer-Primary Subculture Treatment				
Secondary Subculture Treatment (with Carrier)				
Neutralizer-Primary Inoculated Control				
Subculture Subculture Inoculated Control				
Neutralizer-Primary Uninoculated Control Tube				
Subculture Media Uninoculated Control Tube				
*+=growth, 0=no growth				

SUMMARY OF RESULTS: Date/Initials:_____	
Bacteriostatic Effect Observed?	Yes_____ No_____
Comments:	

Neutralization Confirmation Assay: Microbe Confirmation Sheet

OPP Microbiology Laboratory

TEST INFORMATION/Confirmed by:_____			
EPA Reg. No.		Test Date	
Product Name		Test Organism	
Sample No.		Carrier Type	

Source: Tube/Plate ID	Date/ Initials	Stain Results[1]	Media Information			Results		
			Type	Prep. No.	Inc. Time/ Temp.	Date/ Initials	Colony Characteristics	Vitek ID (if applicable)

[1] Record Gram Stain results as GPC=gram positive cocci or GNR=gram negative rods

Neutralization Confirmation Assay Serial Dilution/Plating Tracking Form
OPP Microbiology Laboratory

TEST INFORMATION/Confirmed by:_____			
EPA Reg. No.		Test Date	
Product Name		Neutralizer(s)	
Sample No.		Organism Control #	

Confirmed by:_____	Dilution Tube							
	-1	-2	-3	-4	-5	-6	-7	-8
Vol. In Dil. Tube prior to Addition								
Volume Added to Dil. Tube								
Overall Dilution in Dil. Tube								
Volume Plated								
Overall Dilution on Plate								
Number of Plates per Dilution								
Media Plated Onto								
Comments:								

REAGENT/MEDIA INFORMATION/Confirmed by:_____			
Reagent/Media	Prep No.	Reagent/Media	Prep No.

Neutralization Confirmation Assay Inoculum Enumeration Form
OPP Microbiology Laboratory

TEST INFORMATION/Confirmed by:_____			
EPA Reg. No.		Test Date	
Product Name		Organism	
Sample No.		Test Type	

RESULTS: Date/Initials:_____			
Plating Method			
	CFU per Dilution Plate		Average CFU per mL
Dilution	Plate 1	Plate 2	
1.0×10^{-5}			
1.0×10^{-6}			
1.0×10^{-7}			
1.0×10^{-8}			
TNTC= Too Numerous To Count			
Comments:			

REAGENT/MEDIA INFORMATION/Confirmed by:_____			
Reagent/Media	Prep No.	Reagent/Media	Prep No.